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The induction of respiratory syncytial virus-specific cytotoxic T-cell responses following immunization with a synthetic peptide containing a fusion peptide linked to a cytotoxic T lymphocyte epitope

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SUMMARY

Previously published work has shown that a cytotoxic T-cell epitope (CTL) representing residues 82–90 of the M2 protein of respiratory syncytial virus (RSV) is the target for a protective response against the virus. In this report, we demonstrate that a synthetic peptide representing residues 81–95, when covalently linked to a fusion peptide derived from the conserved N-terminal 19 residues of the F1 protein of measles virus efficiently primes RSV-specific CTLs *in vivo* following immunization without adjuvant.

Cytotoxic T lymphocytes (CTL) play an essential role in eliminating many microbial pathogens.[1] Since inactivated, non-replicating viral vaccines usually fail to induce cytotoxic T-cell responses *in vivo*,[2] new vaccines capable of stimulating CTLs are being developed. The use of immunological adjuvants, such as ISCOMS,[3] saponins,[4] liposomes[5] or Freund's adjuvants[6] can promote the induction of $CD8^+$ CTLs *in vivo* following immunization with protein or peptide antigens. Other methods, such as the modification of synthetic peptide immunogens by linkage to the P3CSS lipopeptide component of *Escherichia coli* have been described as a possible way to stimulate CTL responses *in vivo*.[7]

The use of lipopeptides enables whole proteins and conjugates comprised of the hydrophobic moieties and peptides, to effectively prime CTL responses *in vivo*.[8–10] These responses are, presumably, a consequence of the internalization and introduction of the conjugate into the normal major histocompatibility complex (MHC) class I processing and presentation pathway. The fusion (F) glycoprotein of paramyxoviruses is a type I integral membrane protein involved in virus penetration, haemolysis and cell fusion.[11] The N-terminal 19 residues of F1 are extensively hydrophobic and highly conserved among paramyxoviruses, with up to 90% amino acid sequence identity.[12] In addition, the F peptide can interact with the lipid bilayer of target cells.[13]

In this study, we tested the hypothesis that these N-terminal residues of the F protein can potentiate the induction of CTL responses to a CTL epitope. Immunization of mice with a co-linearly synthesized peptide, comprised of a CTL epitope derived from the M2 protein of respiratory syncytial virus (RSV) (residues 81–95) to the fusion peptide was shown to efficiently induce anti-viral CTL responses *in vivo* in the absence of additional adjuvant.

Four peptides representing previously defined CTL epitopes from the M2 protein of RSV,[14] a measles virus fusion peptide and a chimeric peptide representing M2-9:81-95 and the F peptide were synthesized by the RAMPS (Rapid Multiple Peptide Synthesis; Du Pont, Brussels, Belgium) or by automated solid-phase synthesis (9050 PepSynthesizer, Division of Millipore, MilliGen, Bedford, MA) using Fmoc-chemistry with the f-[2',4'-dimethyloxy phenyl-Fmoc-(amino-methyl)-phenoxy] resin (Novabiochem, Nottingham, UK) (Table 1). Peptides were purified by gel filtration on a Sephadex G-10 column (Pharmacia, Uppsala, Sweden) and by high performance liquid chromatography (HPLC). Six- to 8-week-old female mice ($H\text{-}2^d$) from the National Institute of Medical Research (Mill Hill, London, UK) were injected i.p. or s.c. with 50 nmol of each peptide either in saline or emulsified in Freund's incomplete adjuvant (IFA). Seven to 10 days after a single immunization, spleen cells were restimulated for eight days *in vitro* with 0·5 μM of the 9 amino acid long peptide, M-29:8-90, and 10% ConA supernatant-containing medium (RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine; 10 μg/ml, penicillin; 10 μg/ml, streptomycin; and $5\ 10^{-5}$ M 2-mercaptoethanol) as a source of interleukin-2 (IL-2). Cytolytic activity of *in vitro* secondary CTLs was measured as described previously[15] using P815 $H2^d$ MHC class I^+ class 2^- target cells infected with RSV (A2 strain) at 5 PFU/cell for 18 hr or pulsed with peptide (5 μM) prior to assay.

RSV-specific effector cells from mice intranasally infected with RSV recognized the four M2-derived peptides used to pulse the P815 target cells. Furthermore, lysis of the M2-9-pulsed targets was more effective than lysis of the M2-8 pulsed cells (Fig. 1). Ten days or 4 weeks after immunization with 100 μg of either M2-9:81-95 or M2-8:71-85 in IFA, spleen cells

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Table 1. Notation and sequences of peptide used

Peptide	Sequence
M2-8:71-79	EYALGVVGV
M2-9:82-90	SYIGSINNI
M2-8:71-85	EYALGVVGVLESYIG
M2-9:81-95	ESYIGSINNITKQSA
Fusion peptide	FAGVVLAGAALGVAAAAQI*
M2-9:81-95/Fusion peptide chimera	ESYIGSINNITKQSAFAGVVLA GAALGVAAAAQI

* Reference.[13]

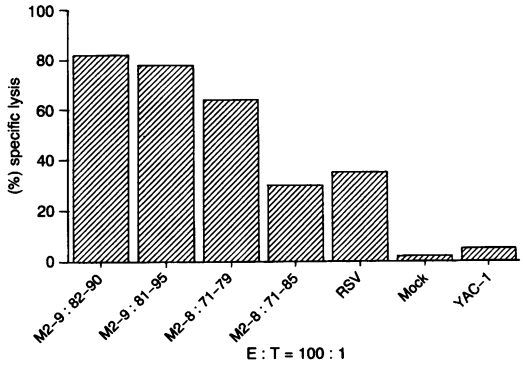

Figure 1. Reactivity of RSV-induced pulmonary CTLs with P815 target cells pulsed with 50 μM M2 protein-derived peptides or pre-infected with RSV (5 PFU/cell). HEp-2 cell debris pulsed P815 targets were used as a control. Effector cells were pulmonary CTLs from mice intranasally infected with RSV-A2 (10^6 PFU/mouse) 7 days previously. In these experiments and others in the paper, YAK cells were used to control for NK cell activity.

were restimulated *in vitro* with 0·5 μM of either M2-0:82-90 or M2-8:71-79 (Fig. 2). Efficient lysis of both M2-9:82-90 pulsed (> 50%) and RSV-infected target cells was observed with M2-9:81-95 activated spleen CTLs. Lower CTL activity was consistently observed with M2-8:71-85 activated spleen cells (Fig. 2b). Only spleen CTLs induced by M2-91:81-95 were able to lyse RSV-infected P815 cells.

To evaluate the ability of the M2-9:81-95/fusion peptide chimera to prime a peptide- or virus-specific CTL response *in vivo*, BALB/c mice were immunized s.c. with 50 nmol of either M2-9:81-95 in saline, M2-9:81-95/fusion peptide chimera in saline, the mixture of M2-9:81-95 and fusion peptide in saline, or M2-9:81-95 in IFA. After 7–10 days, spleen cells were restimulated *in vitro* with 0·5 μM of M2-9: 82–90 (Fig. 3). CTL responses (> 50% at effector: target, 12:1) in spleen cells from mice immunized with the chimera in saline were similar as those induced by M2-9:81-95 emulsified in IFA (Fig. 3).

The class I restricted epitope on the M2 protein of RSV was previously identified[14] using pulmonary $CD8^+$ CTL and assessing the ability of four peptides possessing the K^d specific motif to sensitize target cells for lysis by CTLs from mice infected with RSV or a vaccinia recombinant expressing M2.[14] Resistance to RSV infection in mice, following immunization with the vaccinia-M2 recombinant has recently been shown to be mediated by a single determinant (M2-9:82-90).[16]

The present study confirms and significantly extends this observation to show that RSV-induced pulmonary CTLs lyse target cells pulsed with M2 protein-derived peptides, M2-9:82-90 and M2-8:71-79. Furthermore, 15 amino acid long versions of these peptides (M2-9:81-95 and M2-8:71-85) also sensitize target cells for lysis by RSV-specific pulmonary CTLs (Fig. 1). However, only M2-9:81-95 was able to induce anti-viral CTL responses *in vivo* following immunization in Freund's adjuvant (Fig. 2).

Little is known about the *in vivo* generation of CTL responses following immunization with peptides. In the majority of the cases reported in which synthetic peptides were capable of priming CTLs *in vivo*, the peptides used were relatively long (15 amino acids). It is likely that they also contained T-helper epitopes and usually required the use of additional adjuvants.[15,17–19] The failure of peptides to prime CTL responses *in vivo* could be related to the non-physiological nature of their presentation to the immune system in that CTLs recognize the complex of antigenic peptides and MHC class I molecules endogenously produced in the target cell. In

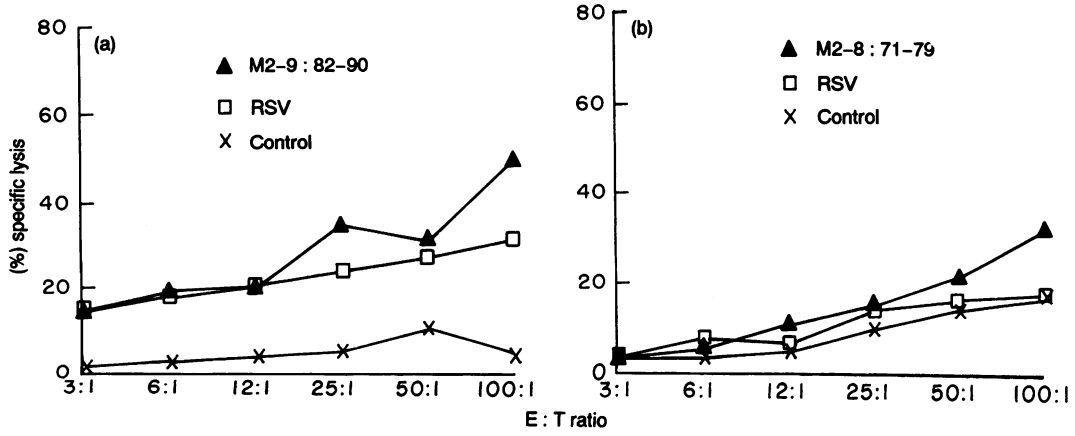

Figure 2. CTL activity in spleen cells following i.p. immunization with (a) M2-9:81-95, (b) M2-8:71-85 emulsified in IFA. P815 target cells were sensitized by (i) pulsing with 5 μM of each nine amino acid long homologous peptide (ii) by infection with RSV at multiplicity of infection of 5 (iii) by incubation with HEp-2 cell debris as a control.

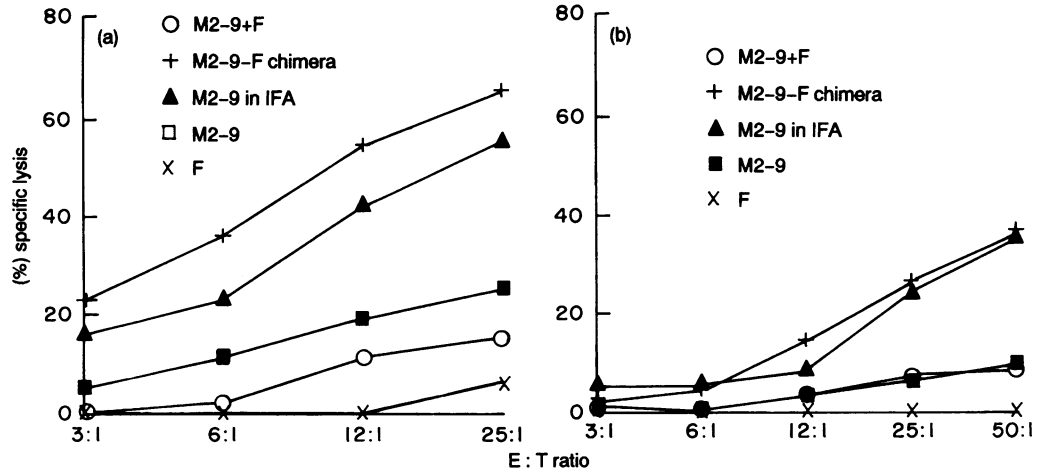

Figure 3. Peptide- or virus-specific CTL responses following s.c. immunization with the fusion peptide (F), M2-9:81-95 (M2-9), a mixture of M2-9:81-95 and fusion peptide (M2-9 + F), and M2-9:81-95/Fusion peptide chimera in saline or M2-9:81-95 emulsified in IFA. P815 target cells were sensitized by (a) pulsing with 5 μM M2-9:82-90 or (b) infection with RSV at a M.O.I. of 5.

addition, this failure could also be due to the absence of exogenous T-cell help.[20–22] In the present study, we have demonstrated that virus-specific CTL responses *in vivo* can be induced by immunization with a chimeric peptide representing a CTL epitope M2-9:81-95 linked to a fusion peptide in the absence of adjuvants. The virus-specific CTL response was as effective as that induced following priming with the CTL epitope M2-9:81-95 emulsified in Freund's adjuvant (Fig. 3).

These data are consistent with those of Hart *et al.*[17] demonstrating CTL activation *in vivo* with a chimeric HIV gp120 synthetic peptide consisting of a class I restricted epitope, SP10MN(A), coupled to a highly hydrophobic peptide representing the fusion domain of gp41 and show tht virus-specific CTL can be induced *in vivo* with a peptide vaccine in the absence of immunological adjuvants.

The mechanism by which the M2-9:81-94/fusion peptide chimera is able to prime CTLs *in vivo* has not been defined. However, the presence of the fusion peptide may extend the *in vivo* half life of the chimera in comparison to that of the free peptides. Attachment of the chimera to the cell membrane via the fusion peptide component may result in efficient entry of the CTL epitope into the cytosol and therefore into the class I restricted antigen-processing pathway. Furthermore, the hydrophobic nature of the chimeric peptide may have allowed its entry into the MHC class I antigen processing pathway, possibly by direct penetration into the cytoplasm or fusion with the plasma membrane.

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